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Inhibitory Effect of BCG Cell-Wall Skeletons (BCG-CWS) Emulsified in Squalane on Tumor Growth and Metastasis in Mice

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The antimetastatic effect of BCG-CWS, which was emulsified in an oil-in-water form with either Drakeol 6VR mineral oil (BCG-CWS/DK) or squalane (BCG-CWS/SQA), on lung metastasis produced by highly metastatic murine tumor cells, Colon26-M3.1 carcinoma cells and B16-BL6 melanoma cells, was investigated in syngeneic mice. An intravenous (i.v.) administration of BCG-CWS (100 mg/mouse) 1 day after tumor inoculation significantly inhibited tumor metastasis of both Colon26-M3.1 carcinoma and B16-BL6 melanoma cells in experimental lung metastasis models. No differences in the antitumor activity of the two oil-based formulations (BCG-CWS/DK and BCG-CWS/SQA) were obverved. However, BCG-CWS/SQA administered through subcutaneous (s.c.) route was shown to be effective only when it was consecutively injected (3 times) after tumor inoculation. An in vivo analysis for tumor-induced angiogenesis shwed that a single i.v. administration of BCG-CWS/SQA inhibited the number of tumor-induced blood vessels and suppressed tumor growth. Furthermore, the multiple administration of BCG-CWS/SQA given at on week intervals led to a significant reduction in spontaneous lung metastasis of B16-BL6 melanoma cells in a spontaneous metastasis model. These results suggest that BCG-CWS emulsified with squalane is a potent inhibitory agent of lung metastasis, and that the antimetastatic effect of BCG-CWS is related to the suppression of tumor growth and the inhibition of tumor-induced angiogenesis.

Key words: bcg-CWS, Squalane, Tumor metastasis, Antitumor effect

INTRODUCTION

A variety of bacterial components are known to possess diverse biological activity that enhances host resistance to cancers (Yoo *et al.*, 2000; Yoshimoto *et al.*, 1976). Heat-killed mycobacteria cells suspended in mineral oil is well recognized as a potent immunoadjuvants, which have been widely used as Freund's complete adjuvant for enhancing both the cell-mediated and humoral immune responses (Freund, 1956). Azuma *et al.* (1971) first reported that the cell-wall skeletons (CWS) were the essential units responsible for the adjuvanticity of mycobacteria. Thereafter, the structure of the *Mycobacterium bovis* BCG cell wall skeleton (BCG-CWS) was determined to be a complex form of "mycolic acid-arabinogaractan-mucopeptide", and the biochemical pro-perties of each component was extensively examined.

In terms of the biological activity of BCG-CWS, the antitumor activity has been demonstrated using transplantable tumor systems in mice, rats and guinea pigs, as well as using autologous tumors in mouse models (Ogura *et al.*, 1978; Tokuzen *et al.*, 1975). BCG-CWS was also shown to possess inhibitory activity on carcinogenesis in various animal models (Hirao *et al.*, 1978; Kuwamura *et al.*, 1977; Yoshimoto *et al.*, 1976).

Since it was shown that the immunological activity of BCG-CWS depends strongly upon the formulation of this adjuvant (Okuyama *et al.*, 1978), BCG-CWS was prepared in the form of an oil-in-water (o/w) emulsion in order to obtain more effective results when applying it to cancer immunotherapy. In fact, BCG-CWS in an o/w emulsion form using Drakeol 6VR successfully induced antitumor activity (Hayashi *et al.*, 1994). However, the

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mineral oil (Drakeol 6VR) had a side effect, which results in pathological changes (Bingham *et al.*, 1967). Consequently, the development of a reliable substitute for Drakeol 6VR, which can guarantee the biological activity of BCG-CWS is needed in order to be applied to human cancer therapy trials.

Metastasis is one of the characteristics that distinguishes malignant tumors from benign neoplasms. Progressive metastasis of tumor cells in the host results in a wide range of biological heterogeneity for immunogenicity, such as growth rate, cell markers and sensitivity to chemotherapeutic drugs along with various biological agents. Therefore, preventing metastasis is one of the most crucial problems in cancer treatment.

Despite the fact that many studies have demonstrated the antitumor activity of BCG-CWS, there are no reports clearly showing that it is not only a safe and effective delivery system for BCG-CWS but it also possesses the antimetastatic activity. In this study, squalane (SQA), a oil obtained by hydrogenating squalene, was examined to determine if it can be substituted for Drakeol 6VR for including the inhibitory activity on the growth and metastasis of highly established metastatic tumor cells in mice. In addition, the mechanisms underlying its antitumor activity were analyzed.

MATERIALS AND METHODS

Animals

Specific pathogen-free female 7-8 weeks old Balb/c and C57BL/6 mice were purchased from the Shizuoka Laboratory Animal Center, Hamamatsu, Japan, and were maintained at either the Laboratory of Animal Experiments, the Institute of Immunological Science, Hokkaido University, Japan, or at the College of Medicine, Konyang University, Korea.

Cells and cell cultures

B16-BL6 melanoma cells were kindly provided by Dr. I. J. Fidler, M. D. Anderson Cancer Center, Houston, Texas, USA. A highly metastatic line of Colon26 carcinoma (Colon26-M3.1) cells were isolated from the lungs of Balb/c mice according to the *in vivo* selection method described by Fidler (1973). Both lung metastatic tumor cells were maintained as monolayer cultures in Eagle's minimal essential medium (MEM) supplemented with 7.5% fetal calf serum, vitamin solution, sodium pyruvate, *nonessential amino acids and L-glutamine.*

Preparation of oil-attached BCG-CWS

The preparation of BCG-CWS was carried out according to the method described previously (Azuma *et al.*, 1974). The constituents related to peptidoglycan, arabinogalactan, and mycolic acid comprised more than 97%. Minimal amounts of phospholipids (less than 0.2%) and amino acids (less than 2%) were included in this preparation, and no mannan and glucose were detected. Due to its insolubility in aqueous and organic solvents, BCG-CWS particles were prepared in an oil-in-water emulsion form as described previously (Azuma and Yamamura, 1979). One mg of BCG-CWS was added to either 5.0 mg of SQA (BCG-CWS/SQA) or 5.2 mg of Drakeol 6VR (BCG-CWS/DK), and the oil-attached particles were suspended in a physiological saline containing 1.1% Tween 80 and 5.6% D-mannitol by grinding in a tissue homogenizer. The suspension was sterilized by heating at 60°C for 30 min.

Experimental lung metastasis

Balb/c or C57BL/6 mice were inoculated i.v. with Colon26-M3.1 carcinoma or B16-BL6 melanoma cells (2.5×10^4/mouse), respectively, and given the indicated doses of BCG-CWS from various routes 1 day after tumor inoculation. The mice were sacrificed 14 days after tumor inoculation. The number of lung tumor colonies was counted using a dissecting microscope after fixing the lung samples in Bouins solution (Yoo *et al.*, 1997).

Spontaneous lung metastasis

C57BL/6 mice were inoculated s.c. with B16-BL6 melanoma cells (5×10^5/mouse) into the right hind footpad, and administered with BCG-CWS from various routes 1, 8, 15, 22 and 29 days after tumor inoculation (Yoo *et al.*, 1997). The primary tumors were surgically removed by amputation 21 days after tumor inoculation. Tumor volume at the time of tumor amputation was calculated by the following formula; $(L \times W^2)/2$, L; long axes, W; width. The mice were sacrificed 35 days after tumor inoculation for the evaluation.

Assay of tumour-induced angiogenesis

The *in vivo* assay of tumor angiogenesis was carried out using a slight modification of the described previously (Yoo *et al.*, 1994). C57BL/6 mice were inoculated intradermally (i.d.) with B16-BL6 melanoma cells (5×10^5/site) at two sites on the back and administered with BCG-CWS (100 μg/mouse) from various routes 1 day after tumor inoculation. Eight days after tumor inoculation, the mice were sacrificed immediately after i.v. injection of 1% Evan's blue solution (200 μl/mouse), and the skin was separated from the underlying tissues. Each of the inoculation

Table I. Effect of BCG-CWS in oil-in-water emulsion on lung metastasis produced by i.v. inoculation of Colon26-M3.1 carcinoma cells

Treatment	Number of lung metastasis	
	Mean ± SD (% inhibition)	Range
Untreated (tumor control)	146 ± 12	(128 - 165)
BCG-CWS (in saline)	169 ± 55	(111 - 229)
BCG-CWS/DK	68 ± 15 (53.4) *,a	(56 - 84)
BCG-CWS/SQA	40 ± 16 (72.6) *,b	(25 - 61)
Vehicle (DK)	128 ± 23	(102 - 149)
Vehicle (SQA)	145 ± 25	(97 - 150)

Groups of five Balb/c mice were inoculated i.v. with 2.5×10^4 Colon26-M3.1 carcinoma cells, and given i.v. injection of BCG-CWS (100 μg/mouse) in SQA or DK o/w
emulsion 1 day after tumor inoculation. Mice were sacrificed 14 days after tumor inoculation for evaluation.
*$p < 0.001$, compared with untreated control (by Student's two-tailed t test)
[a]$p < 0.01$, [b]$p < 0.001$, compared with each vehicle control (by Student's two-tailed t test)

Table II. Effect of BCG-CWS in oil-in-water emulsion on lung metastasis produced by i.v. inoculation of B16-BL6 melanoma cells

Treatment	Number of lung metastasis	
	Mean ± SD(% inhibition)	Range
Untreated (tumor control)	124 ± 20	(99 - 150)
BCG-CWS (in saline)	134 ± 12	(116 - 147)
BCG-CWS/DK	75 ± 15 (39.5) *,a	(57 - 95)
BCG-CWS/SQA	78 ± 19 (37.1) *	(47 - 97)
Vehicle (DK)	116 ± 32	(78 - 159)
Vehicle (SQA)	115 ± 32	(68 - 153)

Groups of five C57BL/6 mice were inoculated i.v. with 2.5×10^4 B16-BL6 melanoma cells, and given i.v. injection of BCG-CWS (100 μg/mouse) in SQA or DK o/w
emulsion 1 day after tumor inoculation. Mice were sacrificed 14 days after tumor inoculation for evaluation.
*$p < 0.01$, compared with untreated control (by Student's two-tailed t test)
[a]$p < 0.05$, compared with each vehicle control (by Student's two-tailed t test)

sites was plated under a dissecting microscope, and the extent of angiogenesis was measured by counting the number of vessels oriented toward the tumor mass. At the same time, tumor size was assessed by averaging the diameter of the short and long axes of the remainder from the injected cells. A single observer made all counts and was blinded to the experiment aims.

Statistical analysis

The statistical significance of difference between the groups was determined by Student's two-tailed *t* test. A p value < 0.05 was considered statistically significant.

Table III. Dose-dependent effect of BCG-CWS on lung metastasis produced by i.v. inoculation of B16-BL6 melanoma cells

Treatment	Dose s(μg)	Number of lung metastasis	
		Mean ± SD (% inhibition)	Range
Untreated (tumor control)		104 ± 12	(84 - 117)
BCG-CWS	500	57 ± 7 (45.2) *	(42 - 69)
	250	61 ± 6 (41.3) *	(44 - 72)
	100	59 ± 10 (43.3) *	(47 - 74)
	50	63 ± 13 (39.4) *	(51 - 77)
	10	98 ± 17	(67 - 119)
Vehicle (SQA o/w)		111 ± 6	(96 - 121)

Groups of five C57BL/6 mice were inoculated i.v. with 2.5×10^4 B16-BL6 melanoma cells, and given i.v. injection of the indicated doses of BCG-CWS in SQA o/w emulsion 1 day after tumor inoculation. Mice were sacrificed 14 days after tumor inoculation for evaluation.
*$p<0.01$, compared with untreated control (by Student's two-tailed t test)

RESULTS

Inhibitory effect of BCG-CWS/SQA on experimental lung metastasis

SQA was first examined to determine if it can act as a substitute for DK and contribute to the antitumor activity of BCG-CWS in an o/w emulsion in experimental lung metastasis models using Colon26-M3.1 cells. As shown in Table 1, i.v. administration of BCG-CWS/SQA (100 μg) 1 day after tumor inoculation significantly reduced lung metastasis of Colon26-M3.1 cells, and the antimetastatic activity of BCG-CWS/SQA was either the same or higher than that of BCG-CWS/DK. Furthermore, BCG-CWS/SQA elicited a therapeutic effect on lung metastasis of B16-BL6 melanoma cells (Table 2). However, BCG-CWS suspended in physiological saline had no effect. This suggests that BCG-CWS emulsified in oil, but not suspended in saline, was able to inhibit the tumor metastasis of both lung metastatic tumor cell lines, Colon26-M3.1 and B16-BL6, and that SQA is also useful for the functional formation of an o/w emulsion of BCG-CWS. As a result, the following experiments were carried out using BCG-CWS/SQA.

Treatment conditions of BCG-CWS/SQA effective for inhibition of tumor metastasis

BCG-CWS/SQA inhibited lung metastasis of B16-BL6 cells in a dose-dependent manner, with a minimal effective dose of 50 μg/mouse (Table 3). In order to examine the effect of the administration route and the frequency of BCG-CWS/SQA on inhibition of tumor metastasis, the mice were given BCG-CWS/SQA once (on day 1) or three (on day 1, 3, 5) times after tumor

Table IV. Effect of single or multiple administration of BCG-CWS on lung metastasis produced by i.v. inoculation of B16-BL6 melanoma cells

Treatment	Route	Injection times	Number of lung metastasis	
			Mean ± SD (% inhibition)	Range
Untreated (tumor control)			128 ± 16	(108 - 146)
BCG-CWS	i.v.	X1	71 ± 7 (44.5) *	(62 - 80)
	i.v.	X3	64 ± 12 (50) *	(49 - 78)
	i.p.	X1	114 ± 18	(98 - 135)
	i.p.	X3	78 ± 6 (31.6) *	(69 - 87)
	s.c.	X1	121 ± 10	(108 - 133)
	s.c.	X3	74 ± 7 (42.2) *	(64 - 83)
Vehicle (SQA o/w)		X1	111 ± 6	(102 - 121)
		X3	108 ± 16	(88 - 128)

Groups of five C57BL/6 mice were inoculated i.v. with 2.5×10^4 B16-BL6 melanoma cells, and given BCG-CWS (100 μg/mouse) in SQA o/w emulsion from the indicated routes after tumor inoculation. Single administration of BCG-CWS was performed on day 1, and multiple administration was on day 1, 3 and 5 after tumor inoculation. Mice were sacrificed 14 days after tumor inoculation for evaluation.
*p<0.01, compared with untreated control (by Students two-tailed t test)

inoculation through various routes, *i.e.* i.v., s.c. or an intraperitoneal (i.p.) injection. Multiple administration of BCG-CWS/SQA significantly inhibited the lung metastasis of B16-BL6 cells regardless of the injection route (Table 4). However, in the case of a single administration, only i.v. injection inhibited tumor metastasis.

Inhibition of spontaneous lung metastasis by BCG-CWS/SQA

The inhibitory effect of BCG-CWS on a spontaneous tumor metastasis was investigated in a spontaneous lung metastasis model in which BCG-CWS/SQA (100 μg) was administered 5 times at an interval of 7 days beginning with the day after the intrafootpad (i.f.) inoculation with B16-BL6 melanoma cells. As shown in Fig.1-A, the multiple administration of BCG-CWS/SQA resulted in a significant inhibition of spontaneous lung metastasis, showing no difference between the i.v. and s.c. injection route. When the inhibitory effect of BCG-CWS on tumor growth was examined 21 days after tumor inoculation, it was found that the multiple administration of BCG-CWS/SQA also distinctively suppressed the growth of the primary tumors (Fig.1-B).

Inhibitory effect of BCG-CWS/SQA on tumor-induced angiogenesis

The effect of BCG-CWS/SQA on the inhibition of tumor-induced angiogenesis was investigated because angiogenesis is an important event for the growth and shedding of metastatic tumors in the primary and metastatic sites, Table 5 shows that the i.v. and s.c. administration of BCG-CWS/SQA 1 day after tumor inoculation resulted in significant inhibition of tumor-induced angiogenesis and the suppression of tumor growth. The results from Fig.1 and 2 indicate that the antimetastatic effect of BCG-CWS/SQA is related to the inhibition of tumor growth and tumor-induced angiogenesis.

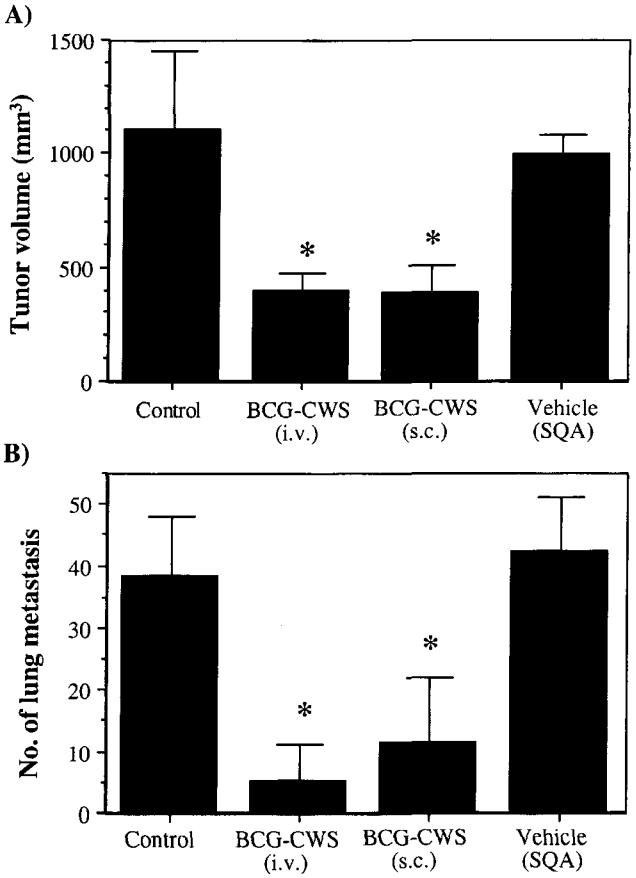

Fig. 1. Inhibitory effect of BCG-CWS on lung metastasis produced by B16-BL6 melanoma cells in a spontaneous metastasis model.
Groups of five C57BL/6 mice were inoculated s.c. with 5×10^5 B16-BL6 melanoma cells into the right hind footpad and were then administered with BCG-CWS/SQA from the indicated routes on day 1, 8, 15, 22 and 29 after tumor inoculation. The primary tumors were surgically removed on day 21, and the mice were sacrificed on day 35 after tumor inoculation for the evaluation. *p < 0.001, compared to the untreated control (by Student's two-tailed *t* test)

DISCUSSION

Bacterial cell wall components have been shown to possess immunomodulating function (Kang *et al.*, 1994) and protective activity against carcinogenesis (Han *et al.*,1999). Since these materials are water-insoluble, the cell wall components such as BCG-CWS and Trehalose-

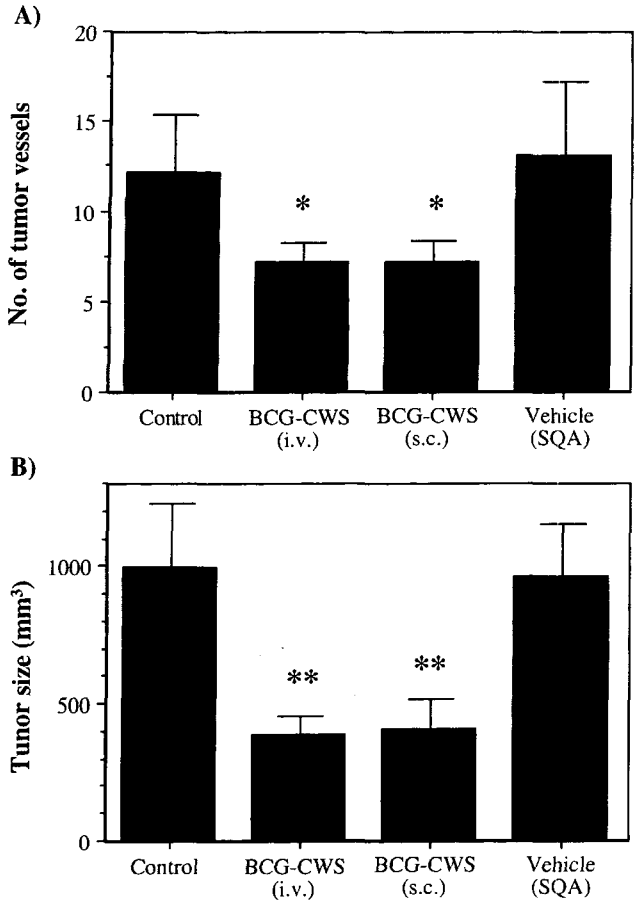

Fig. 2. Inhibitory effect of BCG-CWS on tumor growth and tumor-induced angiogenesis in mice.
Groups of five C57BL/6 mice were given the indicated dose of BCG-CWS/SQA from the indicated routes 1 day after the i.d. inoculation of B16-BL6 cells (5×10^5) at two sites on the back. Seven days after BCG-CWS injection, the mice were sacrificed and the skin was separated from the underlying tissues. Angiogenesis was quantified by counting the number of vessels oriented toward the tumor, and the tumor size was measured. *$p < 0.01$, **$p < 0.001$, compared to the untreated control (by Student's two-tailed *t* test)

6,6'-dimycolate (TDM) are prepared in the form of an o/w emulsion. Mineral oil was generally used as a candidate for the oil vehicle available for o/w emulsion. However, mineral oil has some problems when applied to an oil vehicle due to its complexity, non-sterility and variable composition. In a trial to develop a new oil that is effective in inducing cell-mediated immunity against experimental allergic encephalomyelitis (EAE) in Lewis rats, Whitehouse *et al.* (1974) discovered that squalane, pristane and hexadecane could be excellent substitutes for mineral oil in preparing an adjuvant for inducing EAE. Squalane is obtained by the complete hydrogenation of squalene, which is an intermediate product derived from the biosynthesis of cholesterol, and is found in various tissues of mammals and in some plants. In addition, squalane has a well-known structure and it is considerably digestible. The possibility of squalane to be used as a substitute for mineral oil Drakeol 6VR (DK) was invwstigated. In addition, the antimetastatic effect of BCG-CWS/SQA emulsion was also examined.

A single s.c. administration of BCG-CWS/SQA had a therapeutic effect on inhibiting experimental lung metastasis of two different tumor cells, Colon26-M3.1 and B16-BL6, and showed a similar activity with that of BCG-CWS/DK (Table 1 and 2). Moreover, the multiple administration of BCG-CWS/SQA significantly inhibited the spontaneous lung metastasis and tumor growth of B16-BL6 cells (Fig.1). However, the efficacy of BCG-CWS/SQA in inhibiting tumor metastasis was affected by the injection route and the injection frequency. Intravenous administration was shown to give rise to a stable antitumor activity regardless of its injection frequency (Table 4).

The activity of BCG-CWS in enhancing the cytolytic or cytostatic activity of macrophages against tumor cells was examined in order to analyze the mechanism of the antimetastatic effect of BCG-CWS/SQA. However, BCG-CWS did not activate murine macrophages to kill tumor cells or to suppress the growth of tumor cells, such as 3LL lung carcinoma and B16-BL6 melanoma cells (data not shown). In addition, BCG-CWS neither exhibited direct cytotoxicity against tumor cells nor affected the growth of tumor cells *in vitro* (data not shown). In clinical trials of cancer immunotherapy using BCG-CWS emulsified with DK, IFN-γ production was observed in patients who were being successfully treated and had a good prognosis (Hayashi, 1994). Furthermore, it was shown that the IFN-γ-producing activity of BCG-CWS/DK in patients with lung cancer was related to the activation of blood lymphocytes (Matsumoto *et al.*, 2001). Since a high level of IFN-γ was detected without interleukin-12 in the patients sera after being administered with BCG-CW, BCG-CWS was thought to induce IFN-γ independently of IL-12 production (Hayashi *et al.*, 1996).

The development of a vascular network appears to be associated with the progress of a tumor mass at the primary and metastatic sites and with the process of a metastasis from the original sites to the specific organs. Inhibiting tumor-induced angiogenesis might suppress tumor growth and metastasis. Ogura *et al.* (1978) showed that a treatment with BCG-CWS/DK suppressed tumor cells in a mouse model, and Hayashi (1994) demonstrated the availability of BCG-CWS/DK for cancer immunotherapy in humans. However, it is unclear whether or not the antitumor activity of BCG-CWS is mediated by the inhibition of tumor metastasis. When the inhibitory effect of BCG-CWS/SQA on tumor-induced angiogenesis was examined, the administration of BCG-CWS/SQA after a tumor inoculation significantly reduced the number of

vessels oriented toward the tumor mass and tumor growth (Fig.2). This suggests that the antimetastatic effect of BCG-CWS is partially related to the suppression of tumor growth and tumor-induced angiogenesis. However, further study to determine the precise mechanism involved in the antitumor activity of BCG-CWS is needed.

This study demonstrated that SQA was a good emulsion substitute for mineral oil for BCG-CWS in mouse models. Furthermore BCG-CWS/SQA could inhibit lung metastasis produced by two highly metastatic tumor cells, Colon26-M3.1 and B16-BL6 cells, by suppressing tumor growth and tumor-induced angiogenesis.

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